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SHORT COMMUNICATION

Long-term follow-up of patients treated by adoptive transfer of melanoma tumor-infiltrating lymphocytes as adjuvant therapy for stage III melanoma

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Abstract The first analysis of our clinical trial on interest of using tumor-infiltrating lymphocytes (TIL) as adjuvant therapy for stage III (regional lymph nodes) melanoma was published in 2002 [5]. The aim of this paper is to update clinical results of 7 years of follow-up after the last treated patient. In the trial conducted between December 1993 and January 1999, patients without any detectable metastases after lymph node excision were randomly assigned to receive either TIL plus interleukin-2 (IL-2) for 2 months, or IL-2 only. The duration of the relapse-free interval was the primary objective. Eighty-eight patients were enrolled in the study. Currently, the last analysis performed in June 2006, after a median follow-up of 114.8 months, did not show change of non-significant extension of the relapse-free interval or overall survival. However, this second analysis strengthens our first hypothesis about the relationship between number of invaded lymph nodes and TIL treatment effectiveness. In the group with only one invaded lymph node, the estimated relapse rate was significantly lower ($P_{\text{adjusted}} = 0.0219$) and the overall survival was increased ($P_{\text{adjusted}} = 0.0125$) in the TIL+IL-2 arm compared with the IL-2 only arm. No differences between the two arms, either with regard to the duration of disease-free survival ($P_{\text{adjusted}} = 0.38$) or overall survival ($P_{\text{adjusted}} = 0.43$), were noted in the group with more than one invaded lymph node, whatever the number of invaded lymph nodes. Treatment was compatible with normal daily activity. This study, with a very long follow up (median of almost 10 years), postulates for the first time relationship between TIL efficiency in stage III melanoma (AJCC) and number of invaded lymph nodes, indicating that tumor burden might be a crucial factor in the production of an effective in vitro expansion of T cells specific for autologous tumor antigen, a finding which could be of value in future vaccine development for the treatment of melanoma.

Keywords Adjuvant therapy · Immunotherapy · Tumor-infiltrating lymphocytes · Melanoma

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Introduction

The adoptive immunotherapy for melanoma is a therapeutic approach in which effector cells such as tumor-infiltrating lymphocytes (TIL) with anti-tumor reactivity can be transferred to a patient to mediate the regression of existing tumors. The rationale for this approach is that melanomas are frequently infiltrated by cytotoxic and cytokine-producing $CD8^+$ T cells specific for autologous tumor-associated antigens (TAA) [3, 4].

The group of Rosenberg, before TAA identification, initiated TIL therapy, since these cells were frequently shown to be tumor-reactive [17]. After this first assay, and thanks to the identification of TAA, new trials of TIL transfer,

have been developed and improved for the selection of TIL and the immune follow-up of treated patients. Interestingly, it was retrospectively shown that objective tumor regression was more frequently associated with TAA-specific responses of the injected TIL [11].

The most impressive results in this field (50% response rate) remain those reported by Rosenberg's group in metastatic melanoma patients treated by the infusion of TIL, highly enriched in tumor reactive T cells, and IL-2, following non-myeloablative lymphodepletion [6]. The rational of this preconditioning was based on animal studies suggesting that lymphodepletion was critical for regulatory T cell elimination [17]. Lymphodepletion may also contribute to TIL efficacy by homeostatic mechanisms. This group also performed immunological studies to try to identify TIL properties linked with tumor regressions. They reported that regression correlated with the in vivo persistence of transferred TIL [16]. However, lymphodepletion induced the risk of viral infection or new cancer appearance [7]. Therefore, the critical usefulness of such a treatment must be clearly established.

With the aim to increase clinical efficacy, our group chose to perform TIL ACT trials in an adjuvant setting.

Our study was based on the hypothesis that adjuvant treatment with TIL and s.c. IL-2 could be effective in AJCC stage III (palpable regional lymph nodes) melanoma patients who have not yet shown clinical evidence of metastases. We carried out a randomized open trial to assess treatment with TIL+IL-2 in patients with regional melanoma lymph node metastases, but without any detectable visceral metastases. The primary aim of the study was to check the effect of TIL+IL-2 treatment on relapse-free survival in comparison to IL-2-treated patients and results of the first analysis have been already reported in 2002 with a mean follow up of 4 years [5]. The aim of this short communication is to report the new analysis of our cohort of patients after a mean follow up of 10 years.

Materials and methods

Trial design

Patients aged between 18 and 75 years had to meet the following criteria for inclusion: histologically proven primary cutaneous melanoma without any prior systemic adjuvant therapy; clinically apparent regional lymph node recurrence occurring at any interval after surgery for primary melanoma of any depth (T1-4N recurrent M0); no sentinel node dissection previously carried out; absence of visceral metastases verified by physical examination, chest radiography, liver echography and brain–chest–liver computed-tomography (CT) scan; written patient consent. Contraception was required in women of childbearing age. Randomization was carried out as soon as the histology was confirmed.

After the histology had been confirmed, patients were randomly assigned to receive either two injections of TIL (the first about 6 and the second about 10 weeks post-surgery, according to the duration of the expansion) combined with IL-2 (Proleukin; Chiron) or IL-2 only. IL-2 treatment began 6 weeks after lymph node resection in the control arm at the same doses, 6×10^6 IU/m^2 per day, 5 days a week for 2 weeks, injected subcutaneously. TIL were injected on the same day on which IL-2 treatment was started in the combined arm. One month later, TIL/IL-2 arm patients received the same plan of treatment and control arm patients received IL2 according to the same diagram of the first cycle. After 2 months adjuvant therapy, patients received no other treatment. Only a regular follow-up was performed [5].

The study was a monocentric trial to ensure the reproducibility of lymph node excision. This study was approved by the ethical committee of Nantes (Pays de La Loire).

TIL lines were produced in good manufacturing practice conditions in the unit of cellular and genetic therapy (CHRU, Nantes, France) according to a procedure previously described [10, 14]. Briefly, short term-cultured TIL were isolated by culturing cryopreserved fragments of stage III metastatic lymph nodes into two 12-well tissue culture plates with X-Vivo 15 serum-free medium (Bio*Whittaker, Walkersville, MD, USA) containing 150 U/ml rIL2 (Eurocetus, Rueil-Malmaison, France) and glutamine (1nM) (Bio*Whittaker) for 10–14 days. Therapeutic ex-vivo expanded TIL were derived as follows: 1.8×10^6 short term-cultured TIL were plated at 300 viable lymphocytes/well with irradiated feeder cells (allogeneic PBL and B-EBV cells) into U-bottom microplates in 200 µl of rIL-2 medium. PHA-P (Difco, Detroit, MI, USA) was added on day 0 (15 µg/ml). Ten days later, lymphocytes were recovered from the culture plates, adjusted to 1×10^6 cells/ml in r-IL2 medium and transferred into culture trays for an additional 10 days. The final TIL harvest was performed by centrifuging, washing and suspending the TIL in 4% human serum albumin (LFB, Les Ulis, France). A second TIL expansion was performed, within 1 month from the first one, starting from 1.8×10^6 cryopreserved short term-cultured TIL. Aliquots of TIL suspensions injected to the patients were cryopreserved, to study their tumor specificity, which was done at a later time point, once the autologous tumor cell line had been established in culture. This procedure gives rise to the systematic production of polyclonal TIL lines from each tumor invaded lymph node. However, the autologous melanoma cell lines were successfully established from 40/88 tumor samples (27 from TIL and IL-2 treated patients and 13 from IL-2 only treated

patients). They were established within 6–12 weeks from the same tumor fragments used to derive the TIL.

The total amount of TIL obtained from the first (R1) and second (R2) TIL expansion for each patient varied between 0.21 and 2.7×10^{10} cells.

Flow cytometry analysis confirmed that only T lymphocytes were present in all cases (100% CD3-positive cells).

The 88 TIL expansions (two expansions for each of the 44 patients) were all performed successfully, and then injected into the patients. No technical problems were noted during these expansions and all the bacteriological controls were negative. The polyclonal TIL lines were obtained from tumor-invaded lymph node of each patient randomized to receive TIL treatment combined with IL2, regardless the tumor specificity of infused populations, that was analyzed retrospectively, six/twelve weeks after patient enrollment, time needed to obtain autologous melanoma cell line.

Follow-up

In both treatment groups, clinical examination, full blood counts and biochemical analyses were repeated every 15 days for the first 2 months, then every 2 months for 18 months, and finally every 3 months. Liver echography and brain–chest–liver CT scan were performed every 6 months.

The date and site of first recurrence as well as the date and cause of death were recorded. Adverse events were noted and the WHO toxicity scale was used to grade their severity.

Statistical analysis

The primary and the secondary objectives were respectively the duration of the disease-free interval and overall survival.

The Kaplan–Meier estimates and log rank tests were used for the main efficacy analysis. The log-likelihood ratio test was used to assess different factors. The Cox model was used to adjust treatment comparison on baseline characteristics known to have a prognostic significance, namely: Breslow thickness (<1.5 mm; >1.5 mm), capsular breaking, number of detectable regional nodes (1, >1), sex, age (<50 years, 50 years).

Assumption of proportional hazards was checked for all factors. Interactions between treatment and each prognostic factor were tested. A Bonferroni adjustment was applied to take into account that a significant result could arise at random.

The probability to have at random a significant interaction is lower or equal to 0.05 [8]. For the five interaction tests, a P value of 0.01 or lower was considered to be significant. The probability to have a significant result in the sub group analyses depends on the result of the interaction test. This conditional probability was estimated by simulation of 10,000 clinical trials and divided by 10, number of potential sub group analysis. A threshold of 0.0345 or lower was considered as significant for each sub group analysis.

Results

Long-term analysis

The following analysis was based on data collected in April 2006, when the first and the last patient treated had completed respectively 12 and 7 years of follow-up.

Study population

Eighty-eight patients were enrolled in the study, with 44 subjects in each group; all patients received one of the two treatments and were evaluated. The median follow-up was 114.8 months (range 86.5–145.3 months). The two groups were well balanced for major prognostic factors of melanoma (Table 1).

Disease-free survival

Twenty-nine recurrences were recorded in the TIL+IL-2 group and 32 in the control group. The median for relapse-free interval was 9.8 months for the TIL group and 8.8 months for the control group. This difference was not significant ($P = 0.57$, log rank test) (Fig. 1) as we already shown at the time of the first analysis on June 2000. However, this first analysis showed that the effect of the

Table 1 Patient characteristics according to treatment group

		TIL+IL-2 ($n = 44$)	IL-2 ($n = 44$)
Primary tumor site[a]	Head	7	0
	Trunk	20	22
	Limbs	16	20
	Extremities	1	1
Breslow[b]	<1.5	9 (0.8 ± 0.1)	9 (1.2 ± 0.1)
	>1.5	31 (4.3 ± 0.5)	33 (3.6 ± 0.3)
Invaded nodes	=1	15	19
	>1	29	25
Capsular breaking[c]	Yes	23	22
	No	20	21

[a] Primary tumor site unknown for one IL2 patient

[b] Data not available for four TIL patients and two IL2 patients

[c] Data not available for one TIL patient and one IL2 patient

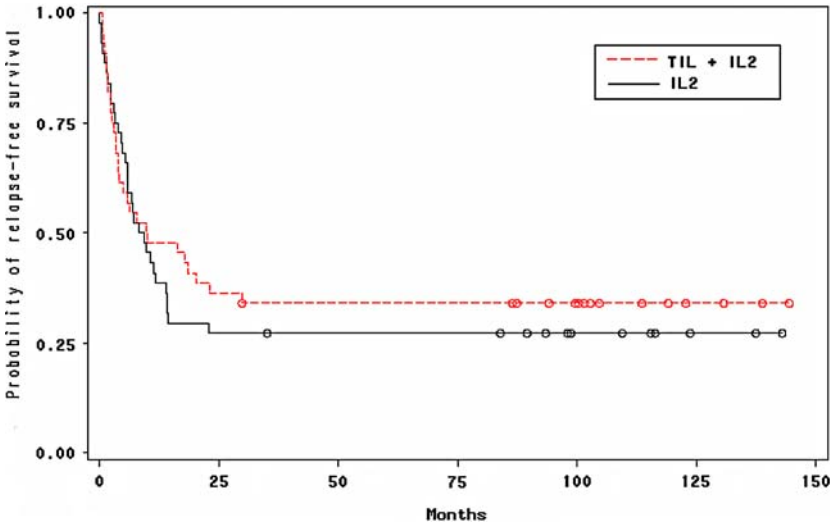

Fig. 1 Relapse-free survival in the TIL+IL-2 group: this difference was not significant ($P = 0.57$; log-rank test); 10 years median follow-up

treatment differed according to the number of invaded lymph nodes (Table 2).

In the group with only one invaded node ($n = 34$), the estimated relapse rate was lower for the patients treated with TIL+IL-2 (5/15) than for the control group (13/19) ($P = 0.0219$) (Fig. 2).

In the group with more than one tumor-invaded node ($n = 54$), the results showed that there were 24 relapses among the 29 TIL+IL2 patients group, and 19 among the 25 patients in the IL-2 control group. As for the results obtained in 2002, the difference was not significant (log rank test; $P = 0.38$; Fig. 3). Moreover, the Cox's model adjusted on the other factors did not show an effect of treatment in the group with more than one invaded node ($\chi^2 = 0.25$; $P = 0.62$). There was no difference between two, three or more invaded lymph nodes. With more than one invaded lymph node, no interaction between TIL and relapse-free survival was noted.

Table 2 Relapse percentage according to treatment and number of invaded nodes (1 or >1)

	TIL+IL-2		IL-2		All	
	2000	2006	2000	2006	2000	2006
1 invaded node	5/15 (33.3%)		13/19 (68.42%)		18/34 (52.94%)	
>1 invaded node	24/29 (82.75%)		18/25 (72%)	19/25 (76%)	42/54 (77.77%)	43/54 (79.6%)
All	29/44 (65.90%)		31/44 (70.45%)	32/44 (72.7%)	60/88 (68.18%)	61/88 (69.3%)

Data obtained in June 2000 and April 2006

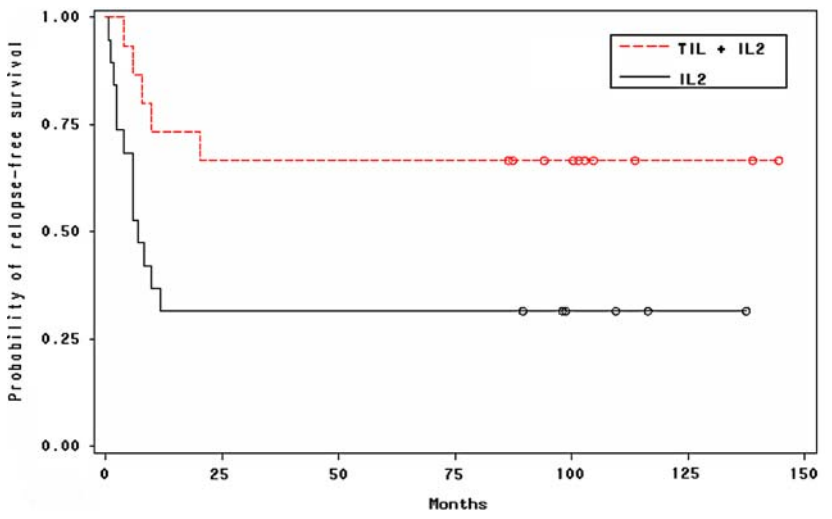

Fig. 2 In the group with only one invaded lymph node, the estimated relapse rate was lower for the patients treated with TIL+IL-2 than for the IL-2 control group ($P_{adjusted} = 0.0219$); 10 years median follow-up

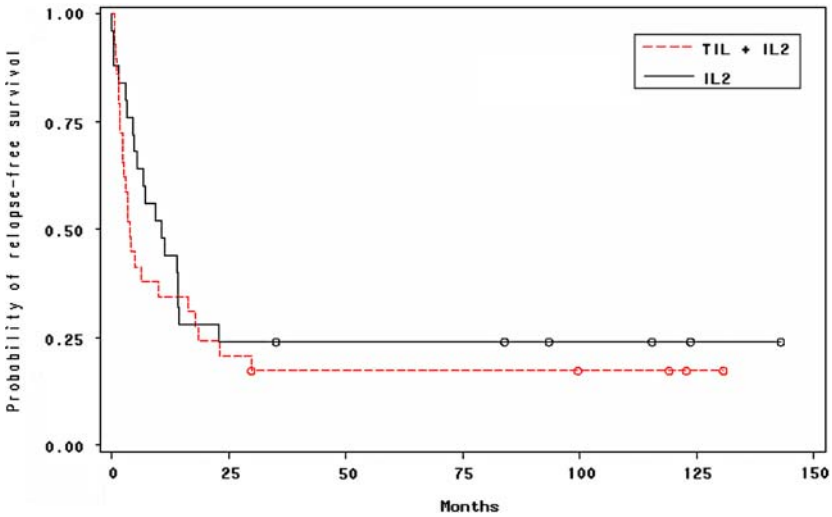

Fig. 3 In the group with more than one invaded lymph node, the estimated relapse rate between the TIL+IL-2 group and IL-2 group was not significant ($P = 0.38$); 10 years median follow-up

Overall survival

Twenty-seven patients died out of the 44 in the TIL+IL-2 group, and 32 out of the 44 in the control group. The median survival time was 32.6 months for the TIL+IL-2 group and 20.5 months for the IL2 control group. This difference was not significant ($P = 0.40$, log rank test; Fig. 4).

As for the relapse-free survival, there was a relationship between treatment and the number of tumor-invaded nodes ($P < 0.01$). Treatment was then assessed in each group of patients by the log-rank test.

In the group with only one tumor-invaded node ($n = 34$), the estimated survival rate was higher for the patients treated with TIL+IL-2 (11/15) than for the control group (6/19) (log rank test; $P = 0.0125$; Fig. 5).

In the group with more than one tumor-invaded node ($n = 54$), there was no difference in survival between the TIL+IL-2 group (6/29) and the IL-2 control group (6/25) (log-rank test; $P = 0.43$; Fig. 6).

Tumor specificity of the TIL

Autologous melanoma lines were obtained from 27 patients treated by TIL and IL-2, and the corresponding ex-vivo expanded TIL were analyzed for their ability to secrete IFN-γ in response to autologous melanoma cells. As reported before [13] and as shown on Table 3, 19 out of 27 patients received TIL containing melanoma reactive T cells. Ten out of 19 patients that had been injected with melanoma-reactive TIL did not relapse, while 7/8 patients that had been injected with preparations devoid of tumor reactive cells did relapse. Kaplan–Meyer statistical analysis shows that relapse-free survival was significantly longer for the group of patients that received tumor reactive TIL ($P = 0.04$).

As mentioned above, we were able to derive systematically TIL lines from each tumor-invaded lymph nodes. TIL-treated Patients ($n = 44$) received between 0.21 and 2.7×10^{10} of TIL through the two infusions, among them 19 patients received between 3×10^6 and 1.12×10^9 tumor

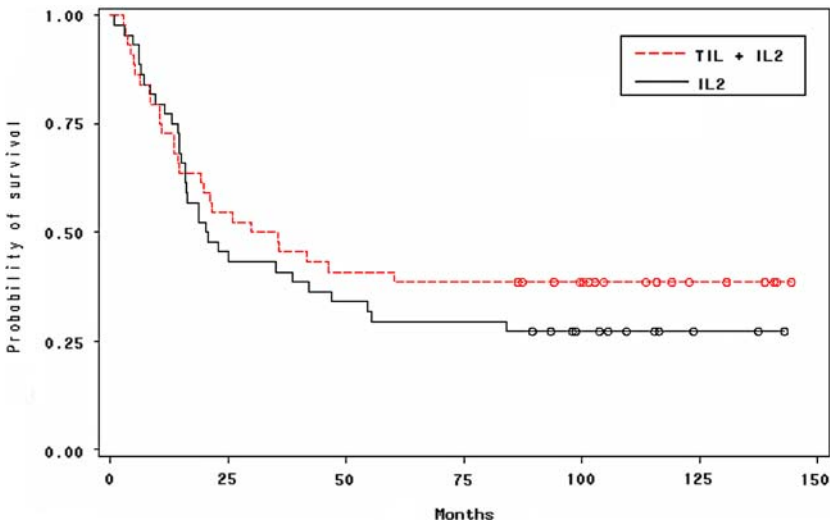

Fig. 4 The overall survival rate between the TIL+IL-2 group and IL-2 group was not significantly different ($P = 0.40$; log-rank test); 10 years median follow-up

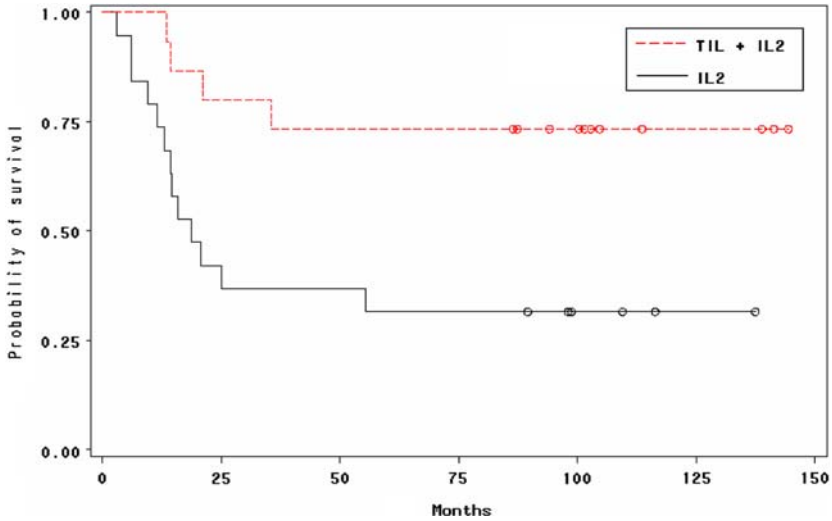

Fig. 5 In the group with only one invaded lymph node, the overall survival rate was greater for the patients treated with TIL+IL-2 than for the IL-2 control group ($P = 0.0125$); 10 years median follow-up

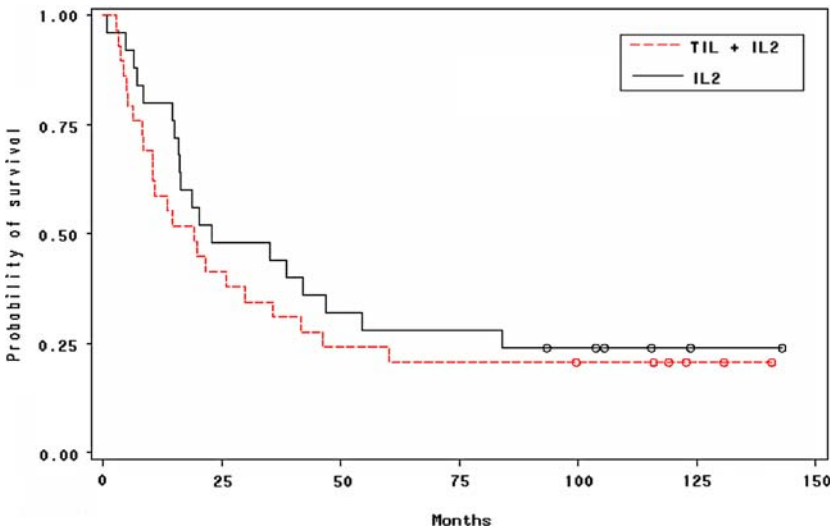

Fig. 6 In the group with more than one invaded lymph node, there was no difference in overall survival between the TIL+IL-2 and the IL-2 control group ($P = 0.43$); 10 years median follow-up

Table 3 Analysis of the presence of tumor specific lymphocytes among TIL infused to 27 patients treated by TIL ± IL-2, according to their clinical status

	Relapse ($n = 16$)	No relapse ($n = 11$)
Presence of tumor specific TIL ($n = 19$)	9	10
Absence of tumor specific TIL ($n = 8$)	7	1

reactive lymphocytes. Despite a significant relationship between the infusion of tumor reactive TIL and a longer relapse-free survival, no correlation could be established between the amounts of total or tumor reactive TIL infused and the clinical status of treated patients.

Safety

All patients treated in this trial experienced at least one adverse effect related to IL-2. Only one patient presented an adverse event, which was related to TIL with a 2-h episode fever, starting 1 h after TIL injection, and spontaneously resolved. IL2 adverse effects were always grade 1 or 2; those usually observed with low doses of IL-2: asthenia (100%), influenza-like symptoms (100%), headache ($n = 78$; 88%), nausea and vomiting ($n = 38$; 43%), dizziness ($n = 21$; 23%), depression ($n = 12$; 14%). No grade 3/4 toxicity or drug-related mortality was observed. No patient withdrawal from the study due to adverse events. No cardiovascular symptoms, haematological toxicity grade 3 or 4, or increase in liver enzyme levels grades 3 or 4 were noted. However, in all patients either erythematic or inflammatory nodules could be observed at the site of the IL-2 injection, which regressed within about 3 weeks.

Concluding remarks

This paper reported the long-term follow up of patients treated by adoptive cell therapy as adjuvant treatment in stage III (AJCC 201) melanoma patients. The previous

published analysis has been performed in 2002 [5]. This second analysis strengthens our first hypothesis about the relationship between number of invaded lymph nodes and TIL treatment effectiveness. After a mean follow-up of 114.8 months, the median survival time remains increased in the TIL+IL-2 group, with 32.6 months compared to 20.5 months in the IL-2 group, but without statistically significant value. Nevertheless, the relationship between the efficiency of TIL transfer and the number of invaded lymph nodes is strongly suggested both for relapse-free survival and overall survival. Indeed, in patients with only one tumor-invaded lymph node, a statistically significant increase in relapse-free survival ($P = 0.0219$) and overall survival ($P = 0.0125$) is always noted in TIL + IL-2 arm compared to the IL-2 arm. In addition the safety remains excellent, without any new side effect with this long-term follow up.

This is the longest follow-up ever reported for melanoma patients stage III treated with TIL adjuvant immunotherapy. This new analysis highlights hypothesis of the potential interest of this treatment at an early stage of the disease, i.e. in patients with only one invaded lymph node.

In recent report, Dudley et al. [6] raise the hypothesis that at metastatic stage (stage IV) the efficacy of adoptive immunotherapy could be limited by regulatory T cells and thus propose a chemotherapy lymphodepletion just before immunotherapy. As it has been reported in other malignancies [12] that the fraction of regulatory T cells could increase upon tumor progression, the efficacy of TIL treatment in one invaded lymph node patient could be related to a lower percentage of Regulatory T cells in these patients.

Furthermore, we recently observed an increased production of TGFβ and IL10 in tumors derived from patients bearing more than one invaded lymph node, two immunosuppressive cytokines susceptible to inhibit activation of infused tumor reactive TIL, by induction of Treg subpopulations [15].

Moreover, we have also established a correlation between the infusion of tumor-specific TIL [13] and of melan-A specific TIL [2] and relapse prevention. The infusion of such specific T cells could be effective only on tumor cells expressing HLA class I molecules and target antigen. Many reports mentioned the emergence of HLA or antigen loss-variants during tumor progression [1, 9, 18]. This could explain the potential effectiveness of TIL treatment at an early stage of disease.

To our knowledge, the long-term effect of an adoptive transfer of TIL melanoma used as an adjuvant regimen for the treatment of stage III melanoma has never been discussed previously. The main result of this first study underlines a hypothesis of the benefit of TIL therapy in stage III melanoma patient with one invaded lymph node. The extent of the disease, which in our study was represented by the number of tumor-invaded lymph nodes, could be a crucial factor in determining the efficiency of TIL treatment.

To evaluate the assumption of the relationship between the TIL efficacy and the number of invaded nodes, we currently perform a multicentric phase III study of TIL infusion to stage III melanoma patients bearing only one invaded lymph node. This ongoing trial will allow us to confirm previous results, to follow antigen specific T cell repertoire in treated patients and to study extensively the impact regulatory T cells on clinical outcome.

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